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Long-term Clomipramine Treatment Upregulates Forebrain Acetylcholine Muscarinic Receptors, and Reduces Behavioural Sensitivity to Scopolamine in Mice

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Abstract

We have investigated the effects of long-term treatment with clomipramine, a tricyclic antidepressant, on central muscarinic acetylcholine receptors (mAChR) in mice.

Repeated clomipramine administration resulted in an increase in the forebrain receptor density value (B_{max}) for [^{3}H]quinuclidinyl benzilate, a muscarinic ligand ($P < 0{\cdot}05$), that was dependent on dose per administration (saline or 5, 10, or 20 mg kg^{-1} once a day for 7 days) and number of days treated (20 mg kg^{-1} for 1, 3, 5, or 7 days). No change in apparent affinity (defined as the reciprocal of the dissociation constant) (K_D) occurred. Seven daily treatments with clomipramine (saline or 5, 10, or 20 mg kg^{-1}) reduced hyperlocomotion induced by scopolamine (0·5 mg kg^{-1}, s.c.) dose-dependently, and the effect of 20 mg kg^{-1} clomipramine was significant ($P < 0{\cdot}05$).

These results suggest that an upregulation of mAChR is produced by repeated clomipramine administration, and such a change is responsible for the decreased sensitivity to the muscarinic antagonist scopolamine.

Tricyclic antidepressant drugs are widely used in the treatment of depression, but their therapeutic effects are typically delayed, requiring long-term administration (Danish University Antidepressant Group 1986; Rudorfer & Potter 1989). The relatively class-specific ability of these agents to inhibit synaptic reuptake of norepinephrine and 5-hydroxytryptamine (5-HT) have been considered essential for their therapeutic effects. In addition to effects on reuptake, tricyclic antidepressants exert blocking activities in several receptor systems (α_1-adrenoreceptors, H_1-histamine receptors, muscarinic acetylcholine receptors); these properties have been linked to side effects (Snyder & Yamamura 1977; Richelson & Nelson 1984; Rudorfer & Potter 1989). In particular, antagonistic actions of antidepressants at muscarinic acetylcholine receptors (mAChR) explain the side effects including dry mouth, constipation, blurred vision, urinary retention, sedation, and memory disturbance. In addition, with long-term use, mAChR may undergo adaptive change that can result in tolerance to anticholinergic side effects, and development of new side effects in association with discontinuation of tricyclic antidepressants (Dilsaver et al 1987). Tricyclic antidepressants are believed to produce upregulation of mAChR, but to our knowledge, this has been demonstrated only with a strong anticholinergic antidepressant, amitriptyline (Majocha & Baldessarini 1984). Furthermore, relationships of dose and number of administrations to this change have been studied insufficiently.

The present study in mice tested the hypothesis that repeated clomipramine administration could also produce an upregulation of mAChR. Clomipramine was chosen because it mainly inhibits 5-HT reuptake in addition to having antimuscarinic properties (Richelson & Pfenning 1984). Siniscalchi et al (1991) have demonstrated that clomipramine reduces cortical acetylcholine release by activating 5-HT_3 receptors. Presynaptic inhibition of acetylcholine release is known to produce

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upregulation of mAChR; upregulation of receptors is generally associated with hypersensitivity to agonists and decreased sensitivity to antagonists (Majocha & Baldessarini 1984). We also suspected that repeated clomipramine administration could reduce behavioural sensitivity to scopolamine. Scopolamine increases ambulatory activity through the activation of the dopaminergic transmission via an antagonistic action at central mAChR (Durkin et al 1983; Kuribara & Tadokoro 1983; Majocha & Baldessarini 1984).

Materials and Methods

Animals

The experimental protocol was approved by the Animal Care and Use Committee of Gunma University School of Medicine. Male ddY mice (Japan Laboratory Animals, Tokyo), five-weeks old and weighing 26–30 g, were used for the experiments. The animals were housed in aluminium cages in groups of 10. They were allowed free access to a solid diet (MF, Oriental Yeast, Tokyo) and tap water. The animal room had a controlled environment (12-h light–dark cycle, lighting period 0600–1800 h; temperature 23 ± 2 °C).

Clomipramine treatments

Clomipramine was dissolved with physiologic saline, and the volume administered was fixed at 0·1 mL/10 g body weight. In the dose–response experiment, mice were given subcutaneous injections of saline (dose = 0) or clomipramine (5, 10, or 20 mg kg^{-1}, daily for 7 days; 10 mice per group). Animals used in the time-course study were treated with 20 mg kg^{-1} clomipramine for 1, 3, 5, or 7 days (5 mice per group). Injections were given between 1100 and 1200 h.

Chemicals

[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, sp. act. 1217 GBq mmol^{-1}) was obtained from Du Pont/NEN Research Products (Boston, MA). Clomipramine HCl, atropine sulphate, pirenzepine dihydrochloride, and scopolamine hydrobromide were purchased from Sigma Chemical Co. (St Louis, MO). The muscarinic antagonist 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AF-DX 116) was generously donated by Boehringer Ingelheim (Ridgefield, CT).

Experimental procedures for the [^{3}H]QNB binding assays

Mice were killed by decapitation approximately 24 h after the last administration of saline or clomipramine. The forebrain was dissected quickly and homogenized in 10 vol ice-cold 50 mM potassium phosphate buffer (pH 7·4) using a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. Samples were centrifuged with an equal volume of ice-cold 50 mM potassium phosphate buffer (pH 7·4) for 20 min at 12 000 *g*. The pellet was rinsed and homogenized, and then recentrifuged for 20 min at 12 000 *g* with ice-cold 50 mM potassium phosphate buffer (pH 7·4). This procedure was repeated and the final membrane pellet was stored at −80 until the time of assay.

Protein concentrations were determined using a protein assay kit (Pierce Laboratories, Rockford, IL) by the bicinchronic acid method, using bovine plasma albumin as a standard (Smith et al 1985).

Saturation binding of [^{3}H]QNB was studied as previously described (Morita et al 1995). Membrane fractions (50 g protein in a total volume of 2 mL) were mixed with 50 mM potassium phosphate buffer (pH 7·4) containing various concentrations of [^{3}H]QNB (10–300 pM). The inhibitory effect of clomipramine on [^{3}H]QNB binding was also compared with that of several muscarinic antagonists (atropine, pirenzepine, or AF-DX116) in a competition–inhibition binding study. Membrane fractions from control mice were incubated with 0·2 nM [^{3}H]QNB in the absence or presence of displacers. After incubation at 30 °C for 1 h, the samples were filtered through Whatman GF/C filters, and immediately washed three times with 3 mL ice-cold 50 mM potassium phosphate buffer (pH 7·4). The filters were placed in plastic minivials, dried, and 3 mL scintillation fluid (Reaflor) added. Radioactivity was measured using an Aloca 650 liquid scintillation counter. Specific binding was defined as the difference between the binding in the presence and in the absence of 1 μM atropine.

Data were subjected to computer-assisted nonlinear regression analysis. Values for receptor density (B_{max}) and affinity (defined as the reciprocal of the dissociation constant, K_D) were obtained by Scatchard analysis of saturation binding data (Scatchard 1949). The displacement curves from competitive binding between [^{3}H]QNB and the various muscarinic antagonists or clomipramine fitted either a one-site model or a two-site model, from which the IC50 for each drug was derived. The IC50 is the concentration of antagonist at which the specific binding of [^{3}H]QNB is 50% of that in the absence of antagonist. The precision of

fit to a one- or two-site model was determined with a partial F-test ($P < 0{\cdot}01$), by comparing the residual sum of squares for fit of data to the respective models (El-Fakahany et al 1986).

Behavioural pharmacological study

One day after the seventh daily administration of clomipramine (5, 10, or 20 mg kg^{-1}) or saline, each mouse was placed in a tilting-type ambulometer having a Plexiglas activity cage 20 cm in diameter (AMB-10, O'Hara & Co., Tokyo) and was allowed 30 min to adapt to the cage. Subsequently the mice were challenge-administered with scopolamine (0·5 mg kg^{-1}, s.c.), followed by quantitation of ambulation. This behavioural experiment was carried out between 1100 and 1500 h using 10 mice per group.

Statistical analyses

Data are expressed as the mean ± s.e.m. Statistical analyses were performed by one-way analysis of variance followed by group comparisons using Scheffe's F-test. A P value less than 0·05 was considered statistically significant.

Results

Saturation binding assays

Forebrain membranes were used in the experiment because several studies have found upregulation of mAChR induced by drugs such as antidepressants, antimuscarinic drugs and ketamine in the forebrain, while change may be difficult to detect in other brain regions (Ben-Barak & Dudai 1980; Majocha & Baldessarini 1984; Morita et al 1995). Daily treatments with clomipramine over the course of one week produced significant dose-dependent increases in B_{max} values without alteration of K_D values (Figure 1A; Table 1). Individual comparisons demonstrated statistically significant changes at doses of 10 and 20 mg ($P < 0{\cdot}05$). Daily administration of 20 mg kg^{-1} clomipramine produced consecutive increases in the B_{max} values in forebrain ($P < 0{\cdot}001$), while having no effect on K_D. These changes were significant on the seventh day of dosing ($P < 0{\cdot}05$) (Figure 1B).

Relative potencies of clomipramine and various muscarinic receptor antagonists for the inhibition of [^{3}H]QNB binding

Nonlinear regression analyses of the data for atropine and clomipramine were best fitted to a one-site model, while data for pirenzepine and AF-DX 116 fitted a two-site model (Figure 2). IC50 values of these displacers for [^{3}H]QNB binding from six independent experiments in duplicate were 6·2 ± 1·0 nM for atropine, 0·27 ± 0·06 μM for pirenzepine, 1·9 ± 0·2 μM for AF-DX 116 and 0·42 ± 0·1 μM for clomipramine. The IC50 value of clomipramine was corrected using the Cheng–Prusoff equation (Cheng & Prusoff 1973), yielding the inhibition constant (K_i). Clomipramine had a

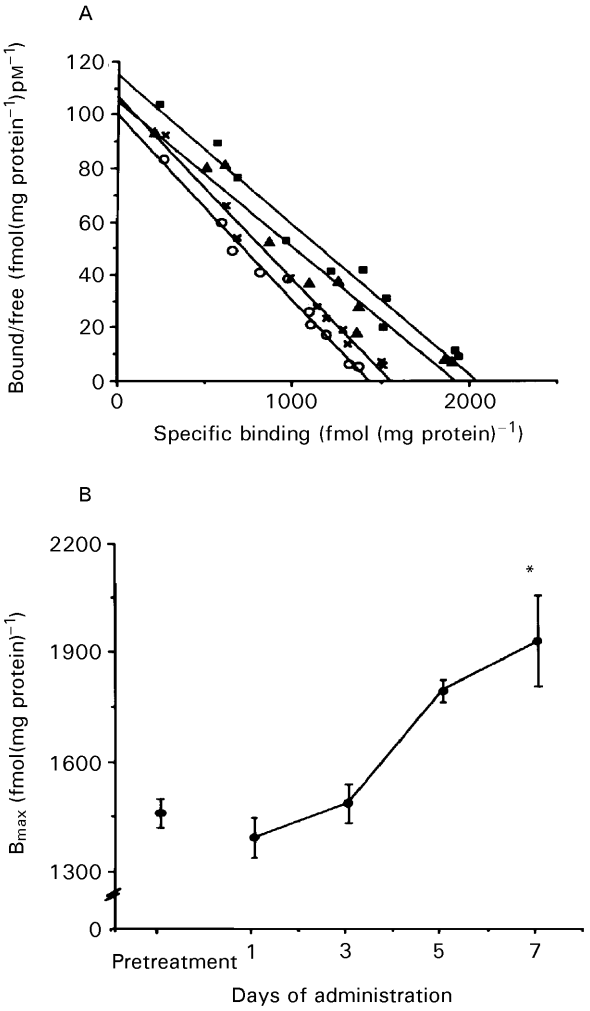

Figure 1. A. Representative Scatchard plots of specific [^{3}H]QNB binding to forebrain membranes after treatment with clomipramine (0: saline, 5, 10, or 20 mg kg^{-1}) once a day for seven days. Repeated clomipramine administration increased B_{max} with no effect on K_D. The data shown are the mean values from duplicate experiments. Other results were similar; the degree of variation is shown in Table 1. Control group, ○; clomipramine 5 ×, 10 ▲, 20 mg kg^{-1} group ■. B. Time-course of changes in [^{3}H]QNB binding during treatment with 20 mg kg^{-1} clomipramine. Daily treatment with 20 mg kg^{-1} clomipramine produced a consecutive upregulation of mAchR in forebrain membranes. These changes became significant at day 7 of treatment, but no changes in K_d were observed. The data shown are the means of five to six independent experiments, with the s.e.m. * $P < 0{\cdot}05$ by Scheffe's F-test, compared with saline treated control.

Table 1. Effects of repeated clomipramine administration on [^{3}H]QNB binding in mice.

Dose of clomipramine (mg kg^{-1})	B_{max} (fmol(mg protein)$^{-1}$)	K_D (pM)
0	1458 ± 34	17·8 ± 0·9
5	1602 ± 37	18·0 ± 1·4
10	1921 ± 78*	19·3 ± 1·9
20	1983 ± 121*	16·6 ± 0·6

Both B_{max} and K_D values for [^{3}H]QNB were obtained by Scatchard plots as described in Materials and Methods. The values are the means ± s.e.m. of six independent experiments. Repeated clomipramine administration produced significant increases in B_{max} for [^{3}H]QNB dose-dependently without any significant changes of K_D. Statistical significance was assessed using one-way analysis of variance followed by Scheffe's F-test (*$P < 0.05$). Mice were treated with clomipramine (0: saline, 5, 10, or 20 mg kg^{-1}) for seven days.

$K_I = 34·3$ nM, which is similar to that (37 nM) reported by Richelson & Nelson (1984) for human brain.

Behavioural pharmacological study

The baseline activity following saline administration to the drug-naive mice was 53 ± 9 counts/1·5 h. The challenge administration of scopolamine produced hyperlocomotion with a peak effect occurring at 20–40 min with a gradual return to baseline within 90 min. Repeated clomipramine administration produced a dose-dependent reduction in scopolamine-induced hyperlocomotion ($P = 0.0380$). Individual comparisons by means of Scheffe's F-test showed statistical significance at the highest dose of clomipramine tested (20 mg kg^{-1}, $P < 0.05$) (Table 2).

Table 2. Behavioural effects of scopolamine (0·5 mg kg^{-1}, s.c.) after repeated clomipramine administration.

Dose of clomipramine (mg kg^{-1})	Ambulatory activity (counts/1·5 h)
0	684 ± 102
5	583 ± 69
10	509 ± 106
20	296 ± 83*

One day after seven days of clomipramine administration (0: saline, 5, 10, or 20 mg kg^{-1}), each mouse was challenge-administered with scopolamine. Measurement of ambulation was carried out for 1·5 h. One-way analysis of variance revealed a dose-dependent reduction in scopolamine-induced hyperlocomotion ($P = 0.0380$). *$P < 0.05$, compared with the 0 mg kg^{-1} group (control). This set of experiments was repeated and similar results were obtained. Each value is the mean ambulatory activity with s.e.m. of ten mice. The baseline activity following saline administration to the drug-naive mice was 53 ± 9 counts/1·5 h.

Discussion

Daily administration of clomipramine produced a dose-dependent- and administration number-dependent increase in density of receptors for QNB. However, no significant change was observed in the K_D values obtained for [^{3}H]QNB binding (Table 1). These results suggest that the binding characteristics of mAChR were similar between controls and the group repeatedly treated with clomipramine. In addition, we showed that daily treatments with clomipramine for seven days reduced behavioural sensitivity of mice to scopolamine.

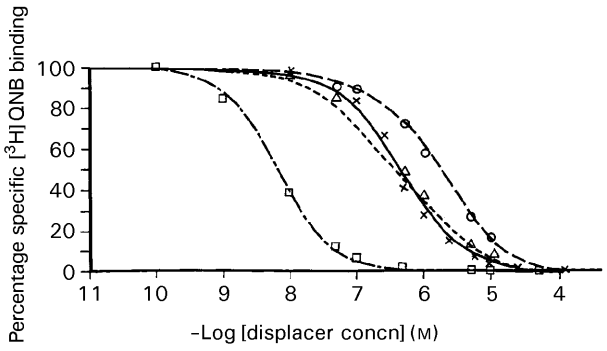

Figure 2. Representative competition–inhibition curves in the forebrain membranes for [^{3}H]QNB binding by atropine (□), pirenzepine (△), AF-DX 116 (○), or clomipramine (X). The data shown are the average values of one experiment in duplicate.

Persistent exposure for agonists has been shown to cause downregulation of mAChR in several systems. On the other hand, upregulation of mAChR is found after long-term administration of muscarinic antagonists, presumably because presynaptically-released acetylcholine is prevented from interacting with the receptor (Hata et al 1980). With long-term use, a strong anticholinergic antidepressant amitriptyline, but not desipramine, was reported to produce upregulation of mAChR (Majocha & Baldessarini 1984). Amitriptyline is ten- to seventeen-times as potent as desipramine (Snyder & Yamamura 1977; Richelson & Nelson 1984). In our competitive inhibition experiment using clomipramine, nonlinear regression analysis of the data was best fitted to a one-site model and the IC50 of clomipramine for [^{3}H]QNB was 0·42 ± 0·1 μM. These data indicate that clomipramine has a high affinity interaction with the [^{3}H]QNB binding site in a manner similar to the classical muscarinic antagonist atropine (McKinney et al 1988). Since the antimuscarinic potency of clomipramine is approximately 50% that of amitriptyline (Richelson & Nelson 1984), the antimuscarinic action of clomipramine is probably at least responsible for this upregulation of mAChR. In addition, clomipramine (10 mg kg^{-1}) is known

to reduce cortical acetylcholine release by activating 5-HT_3 receptors, and the reduction persists even after long-term treatment with clomipramine (Siniscalchi et al 1991). Therefore, this reduction of acetylcholine release should be also pivotal in the upregulation of mAChR observed in this study. However, mAChR are classified into five subtypes with distinct localizations and distinct associated second messenger systems (Jones 1993). The five receptor subtypes can not be distinguished by specific QNB binding. Further study is necessary to clarify the subtype specificity of changes in mAChR and the resulting changes in the second messenger systems.

Scopolamine increases the locomotor activity of rodents by activating dopaminergic transmission through blockade of mAChR within the cortex, striatum, and nucleus accumbens, all of which are located in the forebrain (Durkin et al 1983; Kuribara & Tadokoro 1983; Majocha & Baldessarini 1984). Upregulation of receptors is generally associated with reduction of sensitivity to antagonists. Long-term treatment with scopolamine is known to decrease the sensitivity to scopolamine-induced locomotor stimulation because of upregulation of mAChR (Durkin et al 1983; Kuribara & Tadokoro 1983; Majocha & Baldessarini 1984). In addition, we have shown that upregulation of mAChR induced by repeated ketamine administration was associated with reduction of behavioural sensitivity to scopolamine (Morita et al 1995). Thus, reduced behavioural sensitivity to scopolamine caused by repeated clomipramine treatment observed in the present study may also be related to forebrain mAChR upregulation. This implies that long-term clomipramine administration produces an alteration in acetylcholine transmission. The fact that the upregulation induced by $10\,mg\,kg^{-1}$ clomipramine did not significantly change the behavioural effect of scopolamine may reflect differing sensitivities of binding and behavioural experiments. However, changes in numbers of binding sites were essentially similar for both groups (10 and $20\,mg\,kg^{-1}$ of clomipramine), and might be insufficient to explain reduced sensitivity to scopolamine fully. Even though the behavioural experiment was performed 24 h after the last clomipramine administration, a time compatible with the elimination half-life of the drug, mice receiving $20\,mg\,kg^{-1}$ clomipramine showed some apparent sedation. Accumulation of clomipramine and its metabolites might contribute to reduced sensitivity to scopolamine.

In this study, repeated clomipramine administration produced upregulation of mAChR in the forebrain and reduced the behavioural sensitivity to scopolamine. This adaptive change of mAChR may be an important determinant of the development of tolerance to anticholinergic side effects of clomipramine and may have some secondary modulating influence on its clinical efficacy. However, desipramine, which lacks the ability to upregulate mAChR, is quite effective in the treatment of depression. Thus, this mAChR upregulation is unlikely to be directly related to the therapeutic effects of clomipramine. Rather, it may contribute to withdrawal symptoms suggestive of an alteration in acetylcholine transmission that frequently accompanies cessation of clomipramine treatment (Dilsaver et al 1987).

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References

Ben-Barak, J., Dudai, Y. (1980) Scopolamine induces an increase in muscarinic receptor level in rat hippocampus. Brain Res. 193: 309–313

Cheng, Y., Prusoff, W. H. (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22: 3099–3108

Danish University Antidepressant Group (1986) Citalopram: clinical effect profile in comparison with clomipramine. A controlled multicenter study. Psychopharmacology 90: 131–138

Dilsaver, S. C., Greden, J. F., Michael, R. (1987) Antidepressant withdrawal syndromes: phenomenology and pathophysiology. Int. Clin. Psychopharamacol. 2: 1–9

Durkin, T. P., Hashem-Zaden, H., Villareal, J. E. (1983) Genotype variation in the dopaminergic inhibitory control of striatal and hippocampal cholinergic activity in mice. Pharmacol. Biochem. Behav. 19: 63–70

El-Fakahang, E.E., Cioffi, C.L., Abdellatif, M.M., Miller, M.M. (1986) Competitive interaction of pirenzepine with rat brain muscarinic acetylcholine receptors. Eur. J. Pharmacol. 131: 237–247

Hata, F., Takeyasu, K., Morikawa, Y., Lai, R. T., Ishida, H., Yoshida, H. (1980) Specific changes in the cholinergic system in guinea-pig vas deferens after denervation. Pharmacol. Exp. Ther. 215: 716–722

Jones, S. V. P. (1993) Muscarinic receptor subtypes: modulation of ion channels. Life Sci. 52: 457–464

Kuribara, H., Tadokoro, Y. (1983) Development of tolerance to ambulation-increasing effect of scopolamine dependent on environmental factors in mice. Jpn. J. Pharmacol. 33: 1041–1048

Majocha, R., Baldessarini, R. J. (1984) Tolerance to an anticholinergic agent is paralleled by increased binding to muscarinic receptors in rat brain and increased behavioral response to a centrally active cholinomimetic. Life Sci. 35: 2247–2255

McKinney, M., Lee, N. H., Anderson, D. J., Vella-Rountree, L., El-Fakahany, E. E. (1988) Non-selectivity of amitriptyline for subtypes of brain muscarinic receptors demonstrated in binding and functional assays. Eur. J. Pharmacol. 157: 51–60

Morita, T., Hitomi, S., Saito, S., Fujita, T., Kuribara, H., Uchihashi Y. (1995) Repeated ketamine administration produces upregulation of muscarinic acetylcholine receptors in the forebrain, and reduces the behavioral sensitivity to scopolamine in mice. Psychopharmacology 117: 396–402

Richelson, E., Nelson, A. (1984) Antagonism by antidepressants of neurotransmitter receptors of normal human brain in vitro. J. Pharmacol. Exp. Ther. 230: 94–102

Richelson, E., Pfenning, M. (1984) Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. Eur. J. Pharmacol. 104: 277–286

Rudorfer, M. V., Potter, W. Z. (1989) Antidepressants. A comparative review of the clinical pharmacology and therapeutic use of the 'newer' versus the 'older' drugs. Drugs 37: 713–718

Scatchard, G. (1949) The attraction of protein for small molecules and ions. Ann. N. Y. Acad. Sci. 51: 660–672

Siniscalchi, A., Bianchi, C., Beani, L. (1991) Influence of acute and chronic chlorimipramine treatment on the 5-HT receptor-mediated modulation of acetylcholine release from the cerebral cortex of freely moving guinea pigs. Br. J. Pharmacol. 102: 837–840

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) Measurement of protein using bicinchronic acid. Analy. Biochem. 150: 76–85

Snyder, S. H., Yamamura, H. I. (1977) Antidepressants and the muscarinic acetylcholine receptor. Arch. Gen. Psychiat. 34: 236–239